Genotoxic Effect of Two Bleaching Agents on Oral Mucosa

EVA KLARIC[1], MATEJ PAR[2], IVONA PROFETA[2], NEVENKA KOPJAR[3], RUZICA ROZGAJ[3], VILENA KASUBA[3], DAVOR ZELJEZIC[3] and ZRINKA TARLE[1]

[1]*Department of Endodontics and Restorative Dentistry, School of Dental Medicine, University of Zagreb, Zagreb, Croatia;*
[2]*Private Dental Office, Zagreb, Croatia;*
[3]*Institute for Medical Research and Occupational Health, Zagreb, Croatia*

Abstract. *Aim: To analyze the genotoxic effect of two hydrogen peroxide-containing bleaching products on oral mucosal cells. Materials and Methods: The research was conducted on 22 individuals divided into two groups. Group 1 used ZOOM2 and group 2 the Opalescence BOOST bleaching agent. Specimens of the gingival and the upper lip mucosa were obtained before, immediately after, and 72 h after the bleaching procedure and were analyzed using a micronucleus test. Results: Seventy-two hours after bleaching treatment with BOOST, samples collected from the oral mucosa exhibited a statistically significant increase of all genotoxicity markers, with large effect sizes (Cohen's d>0.8) observed in the total number of micronuclei (MN), number of cells with 3+ MN, karyolysis and bi-nuclear cells. ZOOM2 treatment showed a significant increase, with medium-to-large effect sizes, in the number of cells with 1 MN, karyolysis, nuclear buds and bi-nuclear cells. Conclusion: Both preparations demonstrated potential genotoxic effects.*

An active agent of tooth bleaching preparations is hydrogen peroxide (1, 2). Genotoxic effects of hydrogen peroxide are the result of the formation of free radicals which can damage a number of intracellular structures (3, 4). Previous studies of hydrogen peroxide toxicity were conducted *in vitro* on cell cultures and *in vivo* in animal models. Genotoxicity of hydrogen peroxide has been demonstrated in bacterial cell cultures (5-7), as well as in eukaryotic cell cultures (8, 9). However, the addition of catalase prevented genotoxic effects in cell cultures (10). Genotoxic effects of hydrogen peroxide *in vivo* are questionable – the lack of genotoxic, carcinogenic or promoting activity has been demonstrated in several studies (11-13). However, some studies reported an increased incidence of duodenal hyperplasia and its malignant transformation after long-term oral ingestion of hydrogen peroxide (4-16).

Differences in the results of *in vivo* and *in vitro* studies are probably due to different experimental conditions under which cell cultures were exposed (5-16). Cells *in vitro* are directly exposed to high concentrations of hydrogen peroxide and lack protective mechanisms such as catalase and antioxidants. Moreover, the DNA of bacterial cells is located in the cytoplasm and is hence more susceptible to damage caused by free radicals than is eukaryotic DNA, which is protected by a nuclear membrane and whose integrity is protected by effective repair mechanisms. Furthermore, cells under physiological conditions are not exposed to such high concentrations of hydrogen peroxide and also have a higher activity of enzymes that neutralize free radicals (4). For all these reasons, cells in their natural *in vivo* environment are much more capable of resisting the genotoxic effect of free radicals than are cells in culture.

Previous clinical research of the side-effects of dental bleaching has mostly focused on postoperative sensitivity and gum irritation, while data on genotoxicity are lacking (17). Although gum irritation occurs frequently, it is not considered a risk factor for development of oral cancer (17). There is also no evidence of any cancer-promoting activity of hydrogen peroxide in smokers and people who consume large amounts of alcohol (18).

To date, although scarce, *in vivo* studies have not clearly revealed any genotoxic effect of hydrogen peroxide. Clinical studies of hydrogen peroxide contained in tooth bleaching products are not found in the available literature. Regarding the available data on genotoxicity and carcinogenicity of hydrogen peroxide, the International Agency for Research on Cancer (IARC) 1999 made the following conclusions: there is inadequate evidence in humans for the carcinogenicity of hydrogen peroxide, and there is limited evidence in experimental animals for the carcinogenicity of hydrogen peroxide. Accordingly, the IARC classifies hydrogen peroxide as not classifiable as to its carcinogenicity to humans (18).

Correspondence to: Eva Klaric, Department of Endodontics and Restorative Dentistry, School of Dental Medicine, University of Zagreb, Gunduliceva 5, 10000 Zagreb, Croatia. Tel: +385 14899203, e-mail: eklaric@sfzg.hr

Key Words: Genotoxicity test, hydrogen peroxide, micronucleus test, tooth bleaching, oral mucosa.

The micronucleus test on human oral epithelial cells is a minimally-invasive and relatively simple cytogenetic technique that can detect damage to the genome in primarily exposed cells of living organisms *in vivo*. In recent years, the formation of micronuclei (MN) in oral epithelial cells is increasingly being used as a biomarker of exposure to various genotoxic agents and a significant correlation with the risk of cancer has been demonstrated (19-24).

The aim of this study was to investigate the possible genotoxic effect of two bleaching products. The null hypothesis tested was that there is no genotoxic effect on oral mucosa cells caused by using two different in-office dental bleaching treatments.

Materials and Methods

The study was approved by the Ethics Committee of the School of Dental Medicine, University of Zagreb (study approval no. 565-119). The study was conducted on 22 individuals who volunteered in the procedure of tooth bleaching on the basis of informed consent. The selected individuals were younger people, both males and females, between 18 and 25 years of age (mean=22.8 and SD=1.3 years), without any contraindication for tooth bleaching. They had good oral hygiene, were free of periodontal disease and gingival irritation, had healthy teeth without major restoration and were free of cervical lesions. All patients were non-smokers. Patients were excluded from the study if they were pregnant or nursing, had severely-stained teeth (tetracycline stains, fluorosis, endodontic treatment), and had previously undergone tooth bleaching procedures. Participants were randomly assigned equally to two groups according to the bleaching agent used. In the first group, bleaching was carried out using ZOOM2 (Discus Dental, Culver City, CA, USA) which is based on 25% hydrogen peroxide and is supported by light activation. In the second group, Opalescence BOOST (Ultradent Products, South Jordan, UT, USA) based on chemically activated 38% hydrogen peroxide was used. Cell samples were collected using the swab technique from each participant and from two locations: from the gingival area (the first sample) and from the upper lip lining (the second sample). Each of these samples was collected thrice: immediately before, immediately after and 72 h after the bleaching. One participant dropped out before the end of the experiment and was excluded from the analysis. In total, the analysis included 66 measurements for BOOST and 60 measurements (due to drop-out) for ZOOM2 treatment for each of the genotoxicity markers analyzed.

Prior to collecting the first cell sample, the participants rinsed their mouth with water. The surface layer of dead cells from gingiva in the frontal region was removed using sterile gauze and the sample was collected using a cytological brush. The sample was then stored in a sterile eppendorf tube containing a buffer solution (0.1 M Na2EDTA, 0.02 M NaCl, 0.01 M Tris-HCl; pH 7.0) and refrigerated to +4˚C. Cell samples from the upper lip lining were collected in the same manner. Teeth were cleaned using Proxyt prophylactic paste (RDA 7) (Ivoclar Vivadent, Schaan, Lichtenstein) and the mouth was thoroughly rinsed with water. In the first group, the retractor was set in place, the teeth were dried by air stream and the gums were isolated by protective Liquidam gel (Discus Dental) which was illuminated by the polymerization unit (Bluephase; Ivoclar-Vivadent). A 1 to 2 mm-thick layer of bleaching ZOOM2 gel was applied to the labial surfaces of teeth 14-24 and 34-44 using the brush from the original package. Teeth were illuminated by the light source for 15 min. The application of the gel was repeated three times, each application lasting for 15 min. Upon completion of bleaching, the protective gel and retractor were removed and the mouth was rinsed with water. Cell samples from gums and the upper lip lining were collected once again. Participants were scheduled for an appointment in three days, during which the final cell samples from gums and upper lip lining were collected.

Samples were obtained in the same manner for the second group treated with Opalesence BOOST gel, except that the gums were isolated by protective Opaldam gel (Ultradent Products) and the gel was chemically-, not light-activated. Treatments were performed in the morning at the Department of Endodontics and Restorative Dentistry School of Dental Medicine from February until May.

On the day of collection, samples were submitted to the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Upon delivery, samples were centrifuged at 2000 x*g* for 4 min. The supernatant was removed and the cell precipitate was resuspended in fresh buffer solution (0.1 M Na_2EDTA, 0.02 M NaCl, 0.01 M Tris-HCl, pH 7.0). The centrifuging and rinsing procedure was repeated twice. Resuspended cell sediment was applied to a microscopic glass slide warmed to 37˚C. Preparations were dried at 37˚C for 15 min and then fixed in methanol (80% v/v) at 4˚C for 20 min. Fixed preparations were dried at room temperature and stained in a 5% Giemsa solution for 10 minutes. Stained preparations were analyzed by light microscope at ×1000 magnification. The total number of micronuclei (MN) determined in cell samples was counted, as well as the number of cells with one, two, and more than three MN. The inclusion criteria were: MN was entirely separated from the nucleus or was touching the nucleus while the nuclear edge was clearly visible, and the diameter of the MN was less than one-third of the nuclear diameter and of the same color as the nucleus (21). A total of 2,000 cells were analyzed in each sample. Damaged cells and cells with morphological changes of the nuclei were not included in the analysis. Such cells were recorded separately.

Statistical analysis. Due to the high heteroscedasticity between measurements and non-normality, data were transformed prior to analysis. Positively-skewed data and Box-Cox test indicated that logarithmic transformation was appropriate. A repeated measures ANOVA was applied to the transformed data to assess differences among measurements observed at the baseline, immediately after bleaching and 72 h after bleaching. Deviation of residuals from normality was assessed by the Shapiro-Wilk test and normal probability plot. Huynh-Feldt adjustment was used for univariate effects when the assumption of sphericity had been violated. Mean differences in log-transformed data were transformed back to the original scale for reporting so that the difference between groups is expressed as a ratio of geometric means. If ANOVA residuals significantly deviated from normality, Friedman and Wilcoxon signed rank tests were used instead. Furthermore, comparison of gum and lip measurements was performed by Wilcoxon rank sum test.

The size of the population for sample selection was substantially constrained by the study requirements. Nevertheless, the main aim of the study was to address potentially large differences in values of genotoxicity markers observed in the oral mucosa, as small changes were not considered clinically relevant. The selected sample

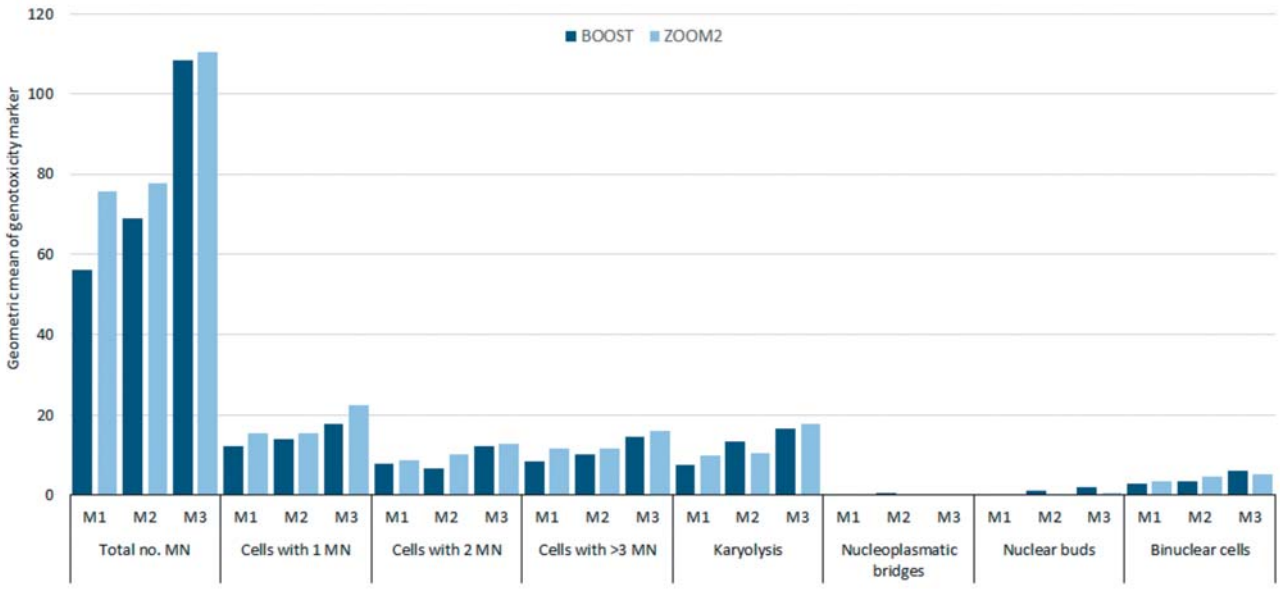

Figure 1. *Comparisons of average values of genotoxicity markers for two different bleaching treatments. M1, Baseline; M2, immediately after bleaching; M3, 72 h after bleaching. MN: micronuclei.*

size was therefore large enough to capture the large effects according to Cohen's effect size conventions (25), *i.e.* Cohen's f=0.40, with a satisfactory statistical power of 80%.

As this was an exploratory study, data were analyzed without multiplicity adjustment (26, 27). The significance level was set to 0.05. Analysis was carried out with SAS System 8.2 (SAS Institute Inc., Cary, NC, USA) and G*Power (Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany).

Results

The average values of genotoxicity markers measured before, immediately after and 72 h after bleaching with BOOST and ZOOM2 are presented in Figure 1. Bleaching led to somewhat higher values of most genotoxicity markers observed. The smallest change was observed in the number of cells with nucleoplasmatic bridges and nuclear buds.

According to the results of repeated measures ANOVA, based on the ratio of geometric means, the total number of MN recorded 72 h after bleaching with BOOST was on average 1.93-times higher compared to baseline ($p<0.001$) and 1.57-times higher compared to the number of MN measured immediately after the bleaching process ($p<0.001$) (Table I). Similar effects, but of a smaller sizes, were also obtained for the number of cells with one MN, two MN and 3+ MN, when bleaching with BOOST led to significant increase in the number of cells after 72 h. The number of cells with one MN was on average 1.45-times higher 72 h after bleaching compared to the baseline ($p=0.012$) and 1.28-times higher compared to the measurements made immediately after bleaching ($p=0.036$). Furthermore, bleaching with BOOST, on average, led to a 1.54- and 1.73-fold increase in the number of cells with two MN and 3+ MN measured 72 h after bleaching compared to baseline ($p=0.007$ and $p<0.001$, respectively) and to a 1.84- and 1.44-fold increase compared to the measurements made immediately after bleaching ($p<0.001$ and $p=0.007$). On the other hand, a significant increase in the number of cells with karyolysis was obtained immediately after bleaching ($p=0.005$) and was 2.22-times higher 72 h after bleaching compared to baseline ($p<0.001$). The number of cells with nucleoplasmatic bridges and nuclear buds also tended to be higher immediately after ($p=0.008$ and $p=0.007$) and 72 h after bleaching ($p=0.043$ and $p=0.003$) compared to baseline measurements, but the observed effects of bleaching were not large in size. Furthermore, treatment with BOOST on average led to 2.12-fold higher number of binuclear cells 72 h after bleaching compared to baseline ($p<0.001$) and was 1.67-times higher compared to values recorded immediately after bleaching ($p=0.002$).

Bleaching with ZOOM2 also significantly affected the values of most genotoxicity markers. The total number of MN was on average 1.42-times higher in measurements recorded 72 h after the bleaching process than immediately after ($p=0.016$) (Table I). A significant effect of bleaching with ZOOM2 was also detected in the number of cells with one MN which was 1.45 times higher 72 h after bleaching compared to baseline ($p=0.007$) and measurements recorded immediately after bleaching ($p=0.013$). Results indicated no statistically significant effect of bleaching with ZOOM2 on

Table I. *The statistical analysis of the effects of two bleaching treatments on genotoxicity markers.*

Marker	Agent	Overall treatment effect[a]		Pairwise comparisons of measurements								
				M2 *vs.* M1			M3 *vs.* M1			M3 *vs.* M2		
		p-Value	Cohen's f	Geometric mean		*p*-Value	Geometric mean		*p*-Value	Geometric mean		*p*-Value
				Ratio	95% CI		Ratio	95% CI		Ratio	95% CI	
Total no. MN	BOOST	<0.001	0.87	1.23	0.92-1.63	0.151	1.93	1.45-2.58	<0.001	1.57	1.37-1.81	<0.001
	ZOOM2	0.045	0.44	1.03	0.78-1.35	0.831	1.46	0.97-2.18	0.066	1.42	1.07-1.87	0.016
Cells with 1 MN	BOOST	0.021	0.45	1.13	0.85-1.52	0.384	1.45	1.09-1.92	0.012	1.28	1.02-1.61	0.036
	ZOOM2	0.009	0.53	0.99	0.75-1.32	0.969	1.45	1.12-1.87	0.007	1.45	1.09-1.93	0.013
Cells with 2 MN	BOOST	<0.001	0.61	0.84	0.58-1.22	0.342	1.54	1.14-2.08	0.007	1.84	1.35-2.49	<0.001
	ZOOM2	0.097	-	-	-	-	-	-	-	-	-	-
Cells with >3 MN	BOOST	<0.001	0.75	1.20	0.95-1.52	0.113	1.73	1.37-2.19	<0.001	1.44	1.12-1.86	0.007
	ZOOM2	0.173	-	-	-	-	-	-	-	-	-	-
Karyolysis	BOOST	<0.001	0.70	1.79	1.21-2.65	0.005	2.22	1.52-3.24	<0.001	1.24	0.87-1.77	0.229
	ZOOM2	0.017	0.55	1.07	0.68-1.69	0.757	1.80	1.12-2.90	0.018	1.68	1.39-2.03	<0.001
Nucleoplasmatic bridges[b]	BOOST	0.017	-	0.50[c]	0.00-1.00	0.008	0.50[c]	0.00-1.00	0.043	–0.50[c]	–0.50-0.50	0.579
	ZOOM2	0.562	-	-	-	-	-	-	-	-	-	-
Nuclear buds[b]	BOOST	0.005	-	1.00[c]	0.00-3.00	0.007	1.50[c]	0.50-3.50	0.003	0.50[c]	–0.50-3.00	0.192
	ZOOM2	0.046	-	0.00[c]	0.00-1.00	0.328	1.25[c]	0.00-2.50	0.016	1.00[c]	0.00-2.00	0.063
B-inuclear cells	BOOST	<0.001	0.89	1.27	0.98-1.64	0.068	2.12	1.64-2.74	<0.001	1.67	1.23-2.27	0.002
	ZOOM2	0.045	0.42	1.35	0.95-1.92	0.086	1.55	1.04-2.31	0.033	1.15	0.82-1.61	0.405

MN: Micronuclei; M1, baseline; M2, immediately after bleaching; M3, 72 h after bleaching. [a]Referring to the differences in the number of cells among three repeated measurements. [b]Friedman test with Wilcoxon signed rank test as a post hoc test applied due to the deviations from normality. [c]Hodges-Lehmann estimate of median treatment difference with corresponding 95% confidence intervals.

the number of cells with two MN and 3+ MN. On the other hand, the number of cells with karyolysis significantly increased 72 hours after bleaching compared to the baseline (1.80-fold increase, $p=0.018$) and measurements made immediately after bleaching (1.68-fold increase, $p<0.001$). Bleaching with ZOOM2 did not induce a significant increase in the number of cells with nucleoplasmatic bridges. However, the number of cells with nuclear buds tended to be higher 72 h after bleaching compared to the baseline measurements ($p=0.016$), but the change was not large. The same pattern was observed for the number of binuclear cells. Bleaching with ZOOM2 on average led to a 1.55-fold higher number of bi-nuclear cells 72 h after bleaching compared to the baseline measurements ($p=0.033$).

Comparison of gum and lip measurements revealed a significant difference in the change of number of cells with one MN from baseline to 72 h after bleaching with BOOST (Wilcoxon rank sum test; $p=0.049$). The median change in the number of cells was 1.0 for gum and 8.0 for lip measurements. Lip measurements also revealed a significantly greater change in the number of cells with two MN recorded immediately after bleaching with BOOST (median=2.0) compared to gum measurements (median=-3.0) (Wilcoxon rank sum test; $p=0.022$). On the other hand, ZOOM2 treatment significantly increased the number of cells with karyolysis between gum and lip measurements. The increase from baseline to 72 h after bleaching was greater for gum (median=8.5) than lip measurements (median=1.0) (Wilcoxon rank sum test; $p=0.030$), respectively.

Discussion

Both bleaching treatments demonstrated a potential genotoxic effect. The null hypothesis that there is no genotoxic effect on oral mucosa cells caused by using two different in-office bleaching treatments was therefore rejected. Nevertheless, the statistically significant increase in the values of genotoxicity markers was relatively small and clinically almost negligible.

Following the bleaching, two samples of cells were collected: the first immediately after bleaching and the second 72 h after bleaching. This allows time for cells to undergo mitosis and the possible genotoxic effect to manifest as an increase in the number of MN. For the formation of MN as a morphological manifestation of genomic damage, a cell must undergo at least one cell cycle. The sample collected immediately after bleaching was used as a control since it was taken about an hour after application of bleaching

preparations. This time is not sufficient for genomic damage to become manifested as the emergence of MN. Accordingly, an eventual increase of MN was expected to be found in the sample taken 72 h after bleaching, whereas in the sample taken immediately after bleaching, such an increase was not expected. Morphological changes in cell nuclei and karyolysis indicate the initiation of molecular mechanisms of cell death. Karyolysis is the morphological manifestation of necrosis. It should be noted that necrosis can be partly induced by mechanical damage to oral cells due to the swabbing technique used. Furthermore, oxidative stress can lead to genomic damage. An increased number of MN suggests that oxidative stress and damage to the genome occurred after bleaching. The number of bi-nuclear cells is an indicator of toxic effects on a cell's protein structures, particularly of the cytoskeleton. Damage to the cytoskeleton affects cytokinesis and normal chromosome migration. As a manifestation of cytokinesis disturbance, cells with two nuclei appear (28). An increased number of binuclear cells implies that damage also affects microtubules of the mitotic spindle, along with other intracellular protein formations. Nucleoplasmatic bridges most commonly occur by fusion of chromosomes damaged in telomeric regions. The appearance of nucleoplasmatic bridges represents a significant disturbance of the integrity of the genome. Nuclear buds are morphological manifestation of the separation of amplified or heavily damaged sections of the genome from the nucleus (19, 29). As statistically significant increase in the number of nuclear buds was detected during BOOST treatment. The observed genotoxic effect on gingival and lip cells may be a result of the direct action of bleaching preparations due to inadequately set gingival protection, or of hydrogen peroxide and its degradation products released from hard dental tissues after completion of the bleaching. The latter is more likely, since no leakage of gingival protection was noticed throughout bleaching treatment.

Furthermore, it is possible that the application of gingival protection gel contributed to the observed increase in the number of MN. Light-curing resin that is used in gingival protection gels contains methacrylate monomers with a potential genotoxic effect (30). As a light-curing resin is never completely polymerized (31), a certain amount of monomer remains in contact with the gingiva for the entire duration of bleaching. The cytotoxic effect of resin composites was demonstrated on several different cells and tissues (32-34).

This study has a number of limitations. High variability present in all three samples (immediately before and immediately after and 72 h after bleaching) was inevitable, since significant differences in the number of MN among the participants were already present in basal conditions. As with many clinical studies, daily food and drink intake, as well as hygienic habits of the individual, were not fully-controlled, relying only on the subjective assessment of respondents. The observed variability is probably a result of exposure to various genotoxic factors in daily life, and reflects the pronounced sensitivity of the MN test manifested as an increase in genotoxicity markers. Fernández *et al*. evaluated the cytotoxicity and genotoxicity of hydrogen peroxide, carbamide peroxide, sodium percarbonate and sodium perborate on mouse fibroblasts and found that all agents exhibited a dose-dependent cytotoxicity. Hydrogen peroxide had the highest cytotoxic and genotoxic effect (35). Ceppi *et al*. analyzed 63 studies which used the MN test to determine the genotoxic effect of various chemicals in occupational or accidental exposure. They concluded that the MN test is a very good minimally-invasive method for monitoring genetic damage in human populations (21). Ionizing radiation plays an important role in the treatment of many neoplasias and several studies evaluated MN in buccal cells and peripheral lymphocytes of patients undergoing radiotherapy in the head and neck. An increase in MN frequency shortly after the initiation of radiotherapy was found (36-38). Lifestyle factors, which include smoking, alcohol consumption, and different types of diet, can also cause an increase in MN frequency (39-41). Today, MN in buccal mucosa cells is used to study pre-neoplastic effects by collecting the cells directly from the affected tissues. MN in buccal mucosa may predict for cancer risk for the upper aerodigestive tract, including pre-malignant stages, such as oral leukoplakia and MN can also be found in patients with diabetes mellitus (42).

It is known that some dental materials increase the frequency of MN (43) and this could also affect the results of the study. In this study the participants were chosen carefully, and five of them had only minor restorations. Therefore, subject selection was one of the most important parts of this study. Goldberg *et al*. reported that in clinical conditions, the daily low-level doses of bleaching product used to produce tooth whitening never generate general acute and sub-acute toxic effects. Genotoxicity and carcinogenicity only occur at concentrations that are never reached during dental treatments (44).

Since no studies evaluating genotoxicity of bleaching preparations in clinical setting by micronucleus test were found in available literature, our results cannot be compared to those of others. As this research was exploratory in nature, the findings should be considered indicative and the corresponding hypothesis should be tested in further confirmatory studies. Our study showed that the bleaching preparations caused certain damage to the genome of the oral mucosal cells, but at this point it is not possible to estimate their actual genotoxic potential. The observed increases of genotoxicity markers, although statistically significant, were relatively small in magnitude, considering high individual variability which exists under basal conditions (21). Since cells of the oral mucosa have a short lifespan, a single exposure to such mild genotoxic noxa probably has

negligible carcinogenic potential. Further research is needed to evaluate the possible genotoxicity and carcinogenicity of bleaching products in clinical setting. Since it is virtually impossible to reduce the variability of genotoxicity markers among respondents in a clinical setting, an increased number of participants is required for a more accurate and detailed assessment, as the results indicate that significant differences between surfaces do exist. Nevertheless, separate analysis of gum and lip was not performed due to the lack of sensitivity, as the study was designed to enable assessment of the influence of bleaching on oral mucosa in general. Separate assessments of gum and lip remain for future study.

Conclusion

Both bleaching preparations demonstrated potential genotoxic effects on oral mucosal cells. The evidence obtained suggests that bleaching treatment affects the genome of mucosal cells to a certain extent, but it is difficult to assess the clinical significance of these findings. Due to the lack of similar studies, it is not possible to discuss the ultimate genotoxic potential of bleaching preparations, although it is probably negligible compared with daily exposure to other genotoxic factors. The patterns presented here provide a framework for ongoing further research, aimed at quantifying the observed effects and their long-term consequences on cells of the oral mucosa.

Authors Contribution

The Authors do not have any financial interest in the companies whose materials are included in this article. The work has not been published before, is not being considered for publication elsewhere and has been read and approved by all Authors. Commercial affiliations as well as consultancies, stock or equity interests, and patent-licensing arrangements which could be considered a conflict of interest are also excluded.

Acknowledgements

This study was supported by the Ministry of Science, Education and Sport, Republic of Croatia (project no. 065-0352851-0410) and Croatian Science Foundation.

References

1 Kwon SR, Ko SH, Greenwall LH, Goldstein RE and Haywood VB: Tooth Whitening in Esthetic Dentistry: Principles and Techniques. London, Quintessence Publishing Co, 2009.
2 Joiner A: The bleaching of teeth: A review of the literature. J Dent *34(7)*: 412-419, 2006.
3 Waris G and Haseeb A: Reactive oxygen species: Role in the development of cancer and various chronic conditions. J Carcinogen *5(1)*: 1-8, 2006.
4 Gamulin S, Marusic M and Kovac Z: Patofiziologija. Zagreb, Medicinska naklada, 2005.
5 Levin DE, Hollstein M, Christman MF, Schwiers EA and Ames BN: A new Salmonella tester strain (TA102) with A X T base pairs at the site of mutation detects oxidative mutagens. Proc Natl Acad Sci USA *79(7)*: 7445-7449, 1982.
6 DeFlora S, Camoirano A, Zanacchi P and Bennicelli C: Mutagenicity testing with TA97 and TA102 of 30 DNA-damaging compounds, negative with other Salmonella strains. Mutat Res *134(4)*: 159-165, 1984.
7 Glatt H: Mutagenicity spectra in *Salmonella typhimurium* strains of glutathione, L-cysteine and active oxygen species. Mutagenesis *4(1)*: 221-227, 1989.
8 Ziegler-Skylakakis K and Andrae U: Mutagenicity of hydrogen peroxide in V79 Chinese hamster cells. Mutat Res *192(1)*: 65-67, 1987.
9 Kruszewski M, Green MHL, Lowe JE and Szumiel I: DNA strand breakage, cytotoxicity and mutagenicity of hydrogen peroxide treatment at 4˚C and 37˚C in L5178Y sublines. Mutat Res *308(2)*: 233-241, 1994.
10 Tsuda H: Chromosomal aberrations induced by hydrogen peroxide in cultured mammalian cells. Jpn J Genet *56(1)*: 1-8, 1981.
11 Li Y, Noblitt T, Zhang A, Origel A, Kafrawy A and Stookey G: Effect of long-term exposure to a tooth whitener. J Dent Res *72*: 248, 1993.
12 Regnier JF, Clare C, de Gerlache J, Malinverno G, Mayr W, Weiner ML and Trochimowicz H: *Ex vivo* and *in vitro* unscheduled DNA synthesis (UDS) assays in rat liver with hydrogen peroxide (H2O2). Mutat Res *379(1)*: 168-169, 1997.
13 Regnier JF, Molinier B, Bentley KS, de Gerlache J, Malinverno G, Mayr W, Weiner ML, Trochimowicz H and Brock W: Micronucleus tests in mice with hydrogen peroxide. Fund Appl Toxicol Suppl *30(1)*: 233, 1996.
14 Ito A, Naito M and Watanabe H: Implication of chemical carcinogenesis in the experimental animal tumorigenic effect of hydrogen peroxide in mice. Nenpo *22(1)*: 147-158, 1981.
15 Ito A, Watanabe H, Naito M and Naito Y: Induction of duodenal tumors in mice by oral administration of hydrogen peroxide. Gann *72(1)*: 174-175, 1981.
16 Ito A, Naito M, Naito Y and Watanabe H: Induction and characterization of gastro-duodenal lesions in mice given continuous oral administration of hydrogen peroxide. Gann *73(1)*: 315-322, 1982.
17 Munro IC, Williams GM, Heymann HO and Kroes R: Tooth whitening products and the risk of oral cancer. Food Chem Tox *44(3)*: 301-315, 2006.
18 International Agency on Research on Cancer (IARC) (1999) Monographs on the Evaluation of Carcinogenic Risks to Humans. Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide. World Health Organization. http://monographs.iarc.fr/ENG/Monographs/vol71/mono71.pdf. Last accessed 17 Jan 2012.
19 Holland N, Bolognesi C, Kircsh-Volders M, Bonassi S, Zeiger E, Knasmüller S and Fenech M: The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat Res *659(1)*: 93-108, 2008.
20 Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S and Zeiger E: HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat Res *534(1-2)*: 65-75, 2003.

21 Ceppi M, Biasotti B, Fenech M and Bonassi S: Human population studies with the exfoliated buccal micronucleus assay: Statistical and epidemiological issues. Mutat Res *705(1)*: 11-19, 2010.
22 Sarto F, Tomanin R, Giacomelli L, Canova A, Raimondi F, Ghiotto C and Fiorentino MV: Evaluation of chromosomal aberrations in lymphocytes and micronuclei in lymphocytes, oral mucosa and hair root cells of patients under antiblastic therapy. Mutat Res *228(2)*: 157-169, 1990.
23 Karahalil B, Karakaya AE and Burgaz S: The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. Mutat Res *442(1)*: 29-35, 1999.
24 Mayer BJ, Laky B, Knasmüller S and Kassie F: Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage in chemopreventive trials. Mutat Res *489(2-3)*: 147-172, 2001.
25 Cohen J: Statistical Power Analysis for the Behavioral Sciences. Second edition. New Jersey: Lawrence Erlbaum Associates, 1988.
26 Corder GW and Foreman, DI: Nonparametric Statistics for Non-Statisticians: A Step-by-Step Approach. New Jersey: Wiley, 2009.
27 Bender R and Lange S: Adjusting for multiple testing – when and how? J Clin Epidemiol *54(4)*: 343-349, 2001.
28 Majno G and Joris I: Apoptosis, oncosis, and necrosis – An overview of cell death. Am J Pathol *146(1)*: 16-19, 1995.
29 Cerqueira EMM, Gomes-Filho IS, Trindade S, Lopes MA, Passos JS and Machado-Santelli G: Genetic damage in exfoliated cells from oral mucosa of individuals exposed to X-rays during panoramic dental radiographies. Mutat Res *562(1-2)*: 111-117, 2004.
30 Lai SC, Tay FR, Cheung GS, Mak YF, Carvalho RM, Wei SH, Toledano M, Osorio R and Pashley DH: Reversal of compromised bonding in bleached enamel. J Dent Res *81(7)*: 477-481, 2002.
31 Knezevic A, Zeljezic D, Kopjar N and Tarle Z: Cytotoxicity of composite materials polymerized with LED curing units. Oper Dent *33(1)*: 23-30, 2008.
32 Imazato S, McCabe JF, Tarumi H, Ehara and Ebisu S: Degree of conversion of composites measured by DTA and FTIR. Dent Mater *17(2)*: 178-183, 2001.
33 Bouillaguet S, Shaw L, Gonzalez L, Wataha JC and Krejci I: Long-term cytotoxicity of resin-based dental restorative materials. J Oral Rehabil *29(1)*: 7-13, 2002.
34 Quinlan CA, Zisterer DM, Tipton KF and Sullivan IO: *In vitro* cytotoxicity of a composite resin and compomer. Int Endod J *35(1)*: 47-55, 2002.
35 Fernández MR, Carvalho RV, Ogliari FA, Beira FA, Etges A and Bueno M: Cytotoxicity and genotoxicity of sodium percarbonate: A comparison with bleaching agents commonly used in discoloured pulpless teeth. Int Endod J *43(2)*: 102-108, 2010.
36 Minicucci EM, Kowalski LP, Maia MA, Pereira A, Ribeiro LR, de Camargo JL and Salvadori DM: Cytogenetic damage in circulating lymphocytes and buccal mucosa cells of head-and-neck cancer patients undergoing radiotherapy. J Radiat Res *46(2)*: 135-142, 2005.
37 Cao J, Liu Y, Sun H, Cheng G, Pang X and Zhou Z: Chromosomal aberrations, DNA strand breaks and gene mutations in nasopharyngeal cancer patients undergoing radiation therapy. Mutat Res *504 (1-2)*: 85-90, 2002.
38 Bindu L, Balaram P, Mathew A, Remani P, Bhattathiri VN and Nair MK: Radiation induced changes in oral carcinoma cells – a multiparametric evaluation. Cytopathology *14(5)*: 287-293, 2003.
39 Van Schooten FJ, Besaratinia A, De Flora S, D'Agostini F, Izzotti A, Camoirano A, Balm AJ, Dallinga JW, Bast A, Haenen GR, Van't Veer L, Baas P, Sakai H and Van Zandwijk N: Effects of oral administration of *N*-acetyl-l-cysteine: A multibiomarker study in smokers. Cancer Epidemiol Biomarkers Prev *11(2)*: 167-175, 2002.
40 Nair U, Obe G, Nair J, Maru GB, Bhide SV, Pieper R and Bartsch H: Evaluation of frequency of micronucleated oral mucosa cells as a marker for genotoxic damage in chewers of betel quid with or without tobacco. Mutat Res *261(3)*: 163-168, 1991.
41 Bloching M, Hofmann A, Lautenschlager C, Berghaus A and Grummt T: Exfoliative cytology of normal buccal mucosa to predict the relative risk of cancer in the upper aerodigestive tract using the MN assay. Oral Oncol *36(6)*: 550-555, 2000.
42 Zúñiga-González GM, Batista-González CM, Gómez-Meda BC, Ramos-Ibarra ML, Zamora-Perez AL, Muñoz-Magallanes T, Ramos-Valdés C and Gallegos-Arreola MP: Micronuclei in diabetes: Folate supplementation diminishes micronuclei in diabetic patients but not in an animal model. Mutat Res *634(1-2)*: 126-134, 2007.
43 Burgaz S, Demircigil GC, Yilmazer M, Ertas N, Kemaloglu Y and Burgaz Y: Assessment of cytogenetic damage in lymphocytes and in exfoliated nasal cells of dental laboratory technicians exposed to chromium, cobalt and nickel. Mutat Res *521(1)*: 47-56, 2002.
44 Goldberg M, Grootveld M and Lynch E: Undesirable and adverse effects of tooth-whitening products: A review. Clin Oral Investig *14(1)*: 1-10, 2010.

Received August 16, 2013
Revised September 29, 2013
Accepted September 30, 2013